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Article

Trehalose Is a Versatile and Long-Lived Chaperone for Desiccation Tolerance

Hugo Tapia[1] and Douglas E. Koshland[1,*]

[1]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA

Summary

Background: Diverse organisms across taxa are desiccation tolerant, capable of surviving extreme water loss. Remarkably, desiccation tolerant organisms can survive years without water. However, the molecular mechanisms underlying this rare trait are poorly understood.

Results: Here, using *Saccharomyces cerevisiae*, we show that intracellular trehalose is essential for survival to long-term desiccation. The time frame for maintaining long-term desiccation tolerance consists of a balance of trehalose stockpiled prior to desiccation and trehalose degradation by trehalases in desiccated cells. The activity of trehalases in desiccated cell reveals the stunning ability of cells to retain enzymatic activity while desiccated. Interestingly, the protein chaperone Hsp104 compensates for loss of trehalose during short-term, but not long-term, desiccation. We show that desiccation induces protein misfolding/aggregation of cytoplasmic and membrane proteins using luciferase and prion reporters. We demonstrate that trehalose, but not Hsp104, mitigates the aggregation of both cytoplasmic and membrane prions. We propose that desiccated cells initially accumulate both protein and chemical chaperones, like Hsp104 and trehalose, respectively. As desiccation extends, the activities of the protein chaperones are lost because of their complexity and requirement for energy, leaving trehalose as the major protector against the aggregation of cytoplasmic and membrane proteins.

Conclusions: Our results suggest that trehalose is both a more stable and more versatile protectant than protein chaperones, explaining its important role in desiccation tolerance and emphasizing the translational potential of small chemical chaperones as stress effectors.

Introduction

Diverse organisms are capable of surviving extreme water loss for remarkably long periods of time. These anhydrobiotes are found across all taxa [1, 2]. In principle, desiccation could impose a number of stresses capable of inflicting lethal damage including protein misfolding/aggregation, hyperosmolarity, hyperoxidation, and DNA damage [3, 4]. Thus, the study of the desiccation tolerance of anhydrobiotes has the potential to provide novel insights into many aspects of stress and stress responses. However, it is unknown whether any of these stresses, alone or in combination, are actually responsible for desiccation-induced death, or which stress effectors in anhydrobiotes actually allow them to prevent or overcome desiccation-induced damage. It is unknown whether the lethal stresses occur during the initial loss of water or whether they persist or continue to accrue during subsequent desiccation or rehydration. Finally, it is unknown if the activity of stress effectors is required during long-term desiccation.

Many anhydrobiotes accumulate high levels of putative stress effectors such as nonreducing disaccharides (trehalose, sucrose) and hydrophilins (short unstructured hydrophilic proteins) [2, 3, 5–7]. Among these potential stress effectors, trehalose has been studied extensively. Trehalose, a nonreducing disaccharide composed of two glucose molecules, is found in almost all anhydrobiotes including the budding yeast, *Saccharomyces cerevisiae* [2, 7, 8]. For many years, the functional importance of trehalose in desiccation tolerance was based on correlative in vitro data. Evidence demonstrating a causative in vivo role was lacking. For example, introduction of trehalose into desiccation-sensitive organisms by either genetic engineering or biochemical perfusion led to only minimal changes in dehydration tolerance, but not desiccation [9–11]. In plants, the weak effect of trehalose on drought resistance has been attributed to indirect effects of carbohydrate alterations and developmental aberrations [12–15]. Finally, in budding yeast, inactivation of *TPS1*, the gene encoding the first enzyme in trehalose biosynthesis, only marginally impacts desiccation tolerance [16, 17]. This lack of a functional role for trehalose during desiccation is confounding, especially considering the widespread distribution of trehalose among most anhydrobiotes and its role in inhibiting excessive protein aggregation of model substrates upon drying [18]. Recently, two studies have suggested an important in vivo role for trehalose in desiccation tolerance of worms and mosquitoes [19, 20]. The more profound role of trehalose in desiccation tolerance in these two diverse organisms suggests that its potential role in desiccation tolerance of budding yeast may have been missed.

What type of stress during desiccation might trehalose serve to mitigate? One potential clue comes from the observation that desiccated worms unable to synthesize trehalose display hallmarks of membrane damage, consistent with a role for trehalose in preserving membrane structure [19]. Other clues come from in vitro and in vivo studies of the stress effector properties of trehalose under aqueous conditions [7, 8, 21–24]. In aqueous in vitro experiments, trehalose can lower the temperature at which lipid chains melt to form a liquid crystalline phase (T_m), stabilize protein folding, and prevent protein aggregation [7, 21]. In vivo, heat shock or nutrient starvation of aqueous yeast cultures causes trehalose to accumulate to up to 15% of the cell's dry mass [25, 26]. The elevated trehalose levels in heat-shocked cells are necessary to preserve folding and prevent aggregation of a model in vivo protein reporter [23]. Extrapolating these results to desiccation suggests that desiccation might also cause proteotoxic stress that might be mitigated by trehalose. However, desiccation has not been shown to induce proteotoxicity in vivo. Furthermore, it is not clear that the stress effector properties of trehalose in aqueous conditions are relevant to desiccation.

Here, we analyze the role of trehalose in desiccation tolerance in yeast. We begin by demonstrating that trehalose is essential for one of the most remarkable properties of desiccation tolerant organisms, their ability to survive long-term desiccation. We exploit this observation to show that a high threshold level of trehalose is needed for desiccation

*Correspondence: koshland@berkeley.edu

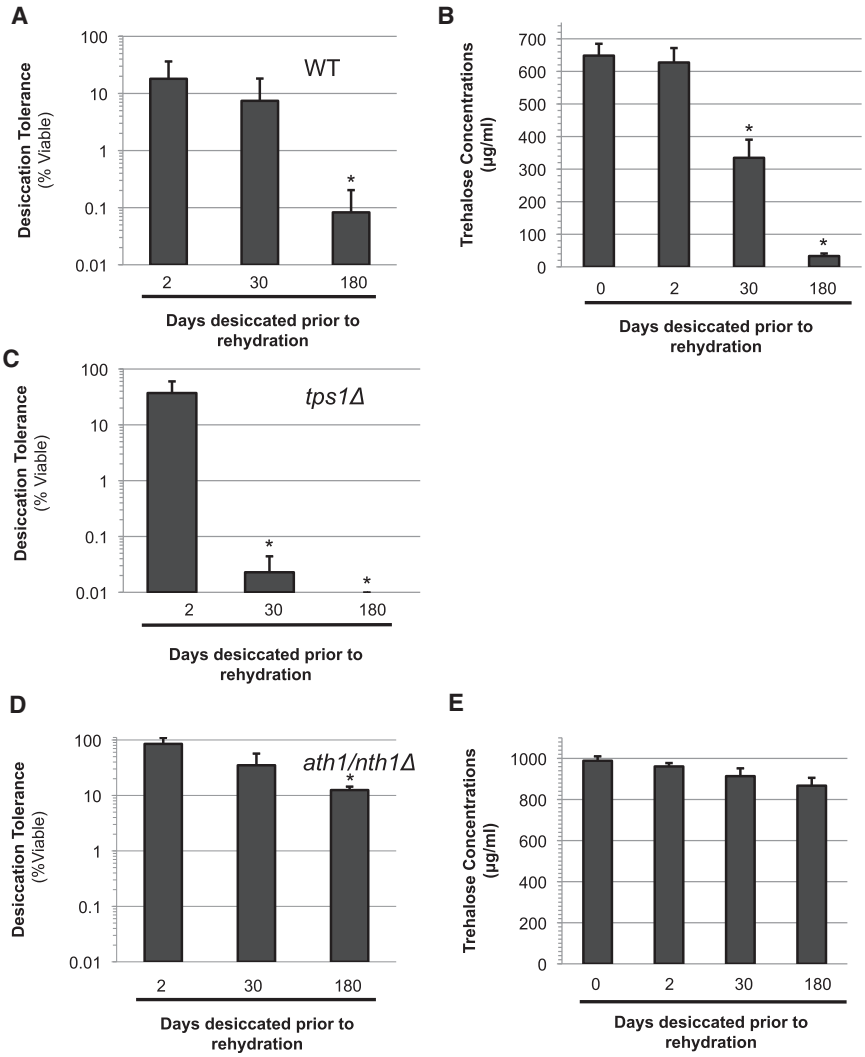

Figure 1. Wild-Type Yeast Lose Viability and Trehalose while in a Desiccated State

Long-term desiccation tolerance assay. Yeast cells were grown to saturation (5 days), air-dried for 2, 30, or 180 days at 23°C, and then rehydrated and assessed for viability by counting colony forming units (cfu).

(A) Long-term desiccation tolerance of wild-type (WT) cells. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 180 day dry sample compared with the corresponding 2 day dry sample.

(B) Intracellular trehalose concentrations of WT cells were assayed over the same long-term dry period. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 30 or 180 day dry sample compared with the corresponding 2 day dry sample.

(C) Long-term desiccation tolerance of *tps1Δ* cells. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 30 or 180 day dry sample compared with the corresponding 2 day dry sample.

(D) Long-term desiccation tolerance of *ath1nth1Δ* cells. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 180 day dry sample compared with the corresponding 2 day dry sample.

(E) Intracellular trehalose concentrations of *ath1Δnth1Δ* cells were assayed over the same long-term dry period. Desiccation tolerance is expressed as the percentage of relative viability, the colony forming unit of the desiccated and rehydrated culture divided by the undesiccated control. Trehalose concentrations are expressed as µg/ml of approximately 10^7 cells.

See also Figures S1 and S2 available online.

tolerance. This high level of trehalose and tolerance is slowly reduced by trehalase activity that persists in the desiccated state. We show that trehalose cooperates with the heat shock factor Hsp104 to promote survival to short-term desiccation and to the secondary stress of heat. Unlike trehalose, Hsp104 is not required for long-term desiccation tolerance. We provide direct evidence that desiccation induces protein misfolding and aggregation using luciferase and prion reporters, and that these proteotoxic effects of desiccation are mitigated by trehalose. Finally, our data suggest that, whereas trehalose and Hsp104 cooperate to promote proteostasis, trehalose is both a more stable and a more versatile chaperone than Hsp104. These properties explain why trehalose plays such a critical and unique role in anhydrobiosis.

Results

A Role of Trehalose in Long-Term Desiccation Tolerance

The surprising observation that eliminating trehalose biosynthesis (*tps1Δ*) does not impair desiccation tolerance of budding yeast has a number of potential explanations [16, 17]. We recognized that yeast cells in the wild often remain in a desiccated state far longer than the few days imposed by published assays of desiccation tolerance. For example, in the California wine region, the dry season lasts approximately 6–7 months [27]. With this in mind, saturated cultures were desiccated for 2, 30, or 180 days and then rehydrated. The viability of cells in wild-type cultures remained high during the first 30 days and then decreased 100-fold during the ensuing 180 days (Figure 1A). Simultaneously, we examined trehalose levels in wild-type cells desiccated for 2, 30, and 180 days. Trehalose levels dropped 2-fold in the first 30 days and an additional 10-fold by 180 days, correlating with the more dramatic decrease in viability at later times (Figure 1B). This correlation was consistent with a requirement for high levels of trehalose for long-term survival to desiccation.

To further test the role of trehalose in long-term desiccation tolerance, we took advantage of mutations in yeast that compromise its synthesis. Trehalose biosynthesis is a two-step process in which glucose-6-phosphate and UDP-glucose are converted to trehalose-6-phosphate by the protein Tps1 [26]. Deletion of *TPS1* (*tps1Δ*) alone results in the complete loss of trehalose biosynthesis (*tps1Δ*) [28]. We subjected *tps1Δ* cultures to long-term desiccation. Viability of the *tps1Δ* strain dropped more than 100-fold during the first 30 days of desiccation compared to only a few fold for wild-type. By 180 days, no *tps1Δ* cells survived (Figure 1C). Thus, trehalose has a critical function for survival to long-term desiccation.

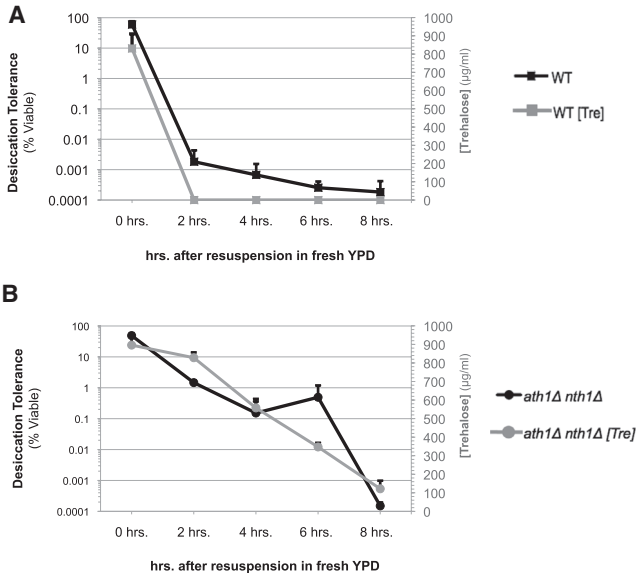

Figure 2. Trehalose Confers Desiccation Tolerance to Logarithmically Dividing Cells

Desiccation tolerance as cells emerge from stationary phase. (A) WT and (B) *ath1Δnth1Δ* cells were grown to saturation in rich media YPD. Cells were released into fresh YPD and assayed for desiccation tolerance and trehalose content every 2 hr for an 8 hr time period.

The decrease in both viability and the concentration of trehalose in wild-type cells was particularly surprising because it occurred during a time when cells were desiccated and presumably lacked any metabolic activity. The decrease in trehalose, albeit slow, could be attributed either to slow environmental decay or enzymatic degradation. Environmental degradation seemed unlikely given the chemical properties of trehalose [29]. We decided to investigate the role of active metabolism in desiccated organisms by inactivating the trehalases responsible for the degradation of trehalose, *ATH1*, and *NTH1* [26]. We assayed a strain lacking both trehalases (*ath1Δnth1Δ*) for its ability to withstand long-term desiccation. In stark contrast to wild-type cells, *ath1Δnth1Δ* cells maintained high levels of viability throughout our 6-month desiccation time course (Figure 1D). Moreover, trehalose levels remained high in the strain lacking the trehalases (Figure 1E). The *nth1Δ*, but not the *ath1Δ*, single mutant also retained desiccation tolerance and high levels of trehalose, indicating that Nth1 is responsible for most of the trehalose breakdown and loss of desiccation tolerance during long-term desiccation (Figure S1). From these results, we conclude that maintaining high levels of trehalose is necessary for long-term desiccation tolerance. The maintenance of high levels of trehalose is countered by the activity of trehalases in desiccated cells. Finally, blocking trehalose metabolism improves desiccation tolerance, indicating that the ability of trehalose to act as a latent energy source is not necessary for desiccation tolerance.

Trehalose might promote long-term survival to desiccation by helping retain residual water in desiccated cells. To address this possibility, we compared the extent of water loss between wild-type and *tps1Δ* cells. Wild-type yeast cells normally have intracellular water content ranging between 65% and 69% w/w [30]. Mutants lacking trehalose retained the same amount of water as wild-type at the onset of desiccation as well as after being desiccated for 1 month (Figure S2). These data indicate that the dramatic decrease in viability of *tps1Δ* cells after 1 month of desiccation cannot be explained by water loss, and trehalose is not required to help cells retain intracellular water. Because trehalose is not directly affecting water content, it must be promoting desiccation tolerance by modulating the function of one or more cellular constituents.

Trehalose Has Overlapping Functions with Hsp104 in Desiccation Tolerance

One way to explain the more dramatic requirement for trehalose in long-term compared to short-term desiccation was to posit the existence of another stress effector(s). This effector protects cells against the same stress(es) of desiccation mitigated by trehalose. The presence of this effector protects cells during short-term desiccation, making trehalose dispensable. However, this second effector has a short half-life making trehalose essential for survival to long-term desiccation.

According to this model, inactivation of the second effector should reveal a role for trehalose in short-term desiccation. Previous studies from our laboratory showed that, 2 hr after saturated cells were rehydrated in fresh nutrients, they lost their tolerance to desiccation, even short-term desiccation [16]. This observation suggests that trehalose and this other putative stress effector must be rapidly inactivated in the presence of fresh nutrients. Indeed, it is known that trehalose levels drop rapidly because of inactivation/downregulation of trehalose biosynthetic enzymes and the activation of trehalases [26]. We reasoned that, by inactivating the trehalases (*ath1Δnth1Δ*), trehalose levels, but not the second effector, would persist longer when saturated cells were diluted into fresh media. If trehalose is important in short-term desiccation, the level of its persistence in freshly diluted cells should correlate with the level of short-term desiccation tolerance.

With this possibility in mind, we diluted saturated cultures of wild-type and the *ath1Δnth1Δ* mutant into fresh media and sampled them at various times for short-term desiccation tolerance and levels of intracellular trehalose. As reported previously, we observed that desiccation tolerance of wild-type cells from a saturated culture was reduced more than four orders of magnitude by 2 hr after rehydration into fresh media (Figure 2A, black line) [16]. Trehalose levels also dropped to undetectable levels over this period of time (Figure 2A, red line). In contrast, trehalose levels slowly decreased over 8 hr in the *ath1Δnth1Δ* mutant (Figure 2B, red line). This slow decrease was likely due to the downregulation of trehalose biosynthesis and subsequent dilution of existing trehalose by cell division [26]. Importantly, prolonging high trehalose levels was accompanied by a dramatic persistence of desiccation tolerance, approximately three orders of magnitude greater than wild-type cells for the first 6 hr (Figure 2B, black line). Interestingly, the levels of trehalose between 6 and 8 hr dropped only 2- to 3-fold, but desiccation tolerance dropped 1,000-fold. The correlation between small changes in trehalose concentration and large changes in desiccation tolerance was very similar to that seen in long-term desiccation (Figure 1). These results reveal a role of trehalose in tolerance to short-term desiccation. They are consistent with our model that this function is masked by an overlapping stress effector(s) that is inactivated by return to growth or long-term desiccation.

A reasonable candidate for one of these putative stress effectors was the heat shock protein Hsp104. Hsp104 has the ability to solubilize aggregated proteins [31, 32]. Hsp104 and trehalose are induced by heat shock, and functional studies

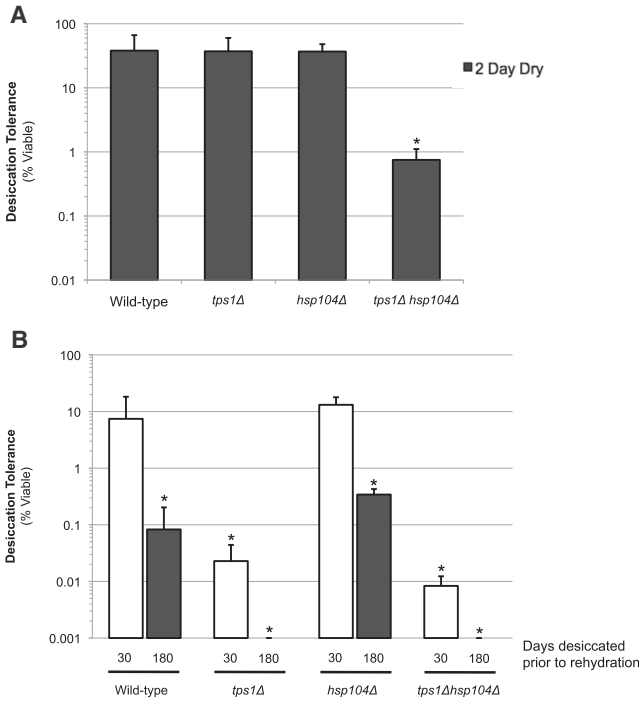

Figure 3. Trehalose Cooperates with Hsp104 for the Establishment of DT, but Not Its Maintenance

Contribution of trehalose and Hsp104 to desiccation tolerance.
(A) Saturated cultures of strains with single (*tps1Δ*, *hsp104Δ*) and multiple gene deletions (*tps1Δhsp104Δ*) were assayed for desiccation tolerance after 2 days of air drying. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the *tps1Δhsp104Δ* sample compared with the corresponding wild-type sample.
(B) Long-term desiccation tolerance of wild-type, *hsp104Δ*, *tps1Δ*, and *tps1Δhsp104Δ*. Desiccation tolerance is expressed as the percentage of relative viability, the colony forming unit of the desiccated and rehydrated culture divided by the undesiccated control. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 30 or 180 day dry sample compared with the corresponding 2 day dry sample.

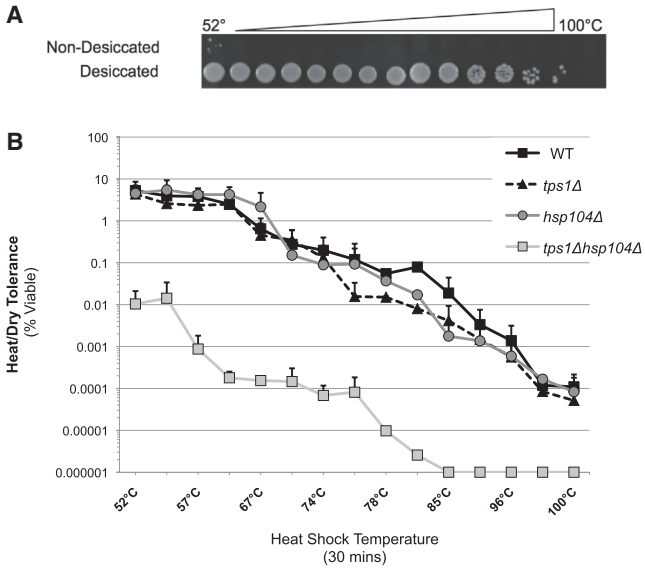

Figure 4. Trehalose Cooperates with Hsp104 to Protect against Secondary Heat Stress while Desiccated

Effect of desiccation on heat tolerance. Samples were prepared and analyzed as described for desiccation tolerance assay. Cells were placed into a vacuum desiccator producing 135 kPa of vacuum overnight. After desiccation, the samples were placed in a thermal cycler and heat shocked at temperatures ranging from 55° to 100°C for 30 min.
(A) Heat tolerance of saturated nondesiccated versus desiccated cells. A 10-fold serial dilution was spotted onto rich media after heat shock.
(B) Saturated cultures of strains with single and multiple gene deletions were dried and assayed for heat tolerance.

suggest they cooperate to mitigate proteotoxicity in aqueous conditions [23, 31, 32]. This connection was particularly intriguing to us because our previous study functionally linked desiccation with heat shock [33].

If Hsp104 was the second effector in our model, then several predictions could be tested. First, Hsp104 should have no effect on short-term desiccation tolerance because of the presence of trehalose. Indeed, an HSP104 deletion (*hsp104Δ*) had wild-type levels of desiccation tolerance (Figure 3A). However, removal of both trehalose and Hsp104 should cause increased sensitivity to short-term desiccation. Indeed, the mutant strain *tps1Δhsp104Δ* exhibited a 50- to 100-fold drop in viability after 2 days of desiccation (Figure 3A). This result indicates that trehalose and Hsp104 cooperate to promote survival from short-term desiccation.

In our model, the activity of the second effector, putatively Hsp104, must decay with time leaving trehalose as the sole protector against the common stress. This model predicts that Hsp104 should not play a role in long-term survival to desiccation. Indeed, an *hsp104Δ* single mutant, unlike *tps1Δ*, showed the same decay in tolerance to long-term desiccation as wild-type (Figure 3B). Furthermore, the introduction of *hsp104Δ* into *tps1Δ* cells did not further enhance the sensitivity to long-term desiccation. The *tps1Δhsp104Δ* cells showed reduced survival to short-term desiccation, but, after this initial drop in survival, the decay in survival to long-term desiccation was similar to *tps1Δ* (Figure 3B). In summary, these observations fit a model in which Hsp104 and trehalose initially cooperate as stress effectors, but with time trehalose functions alone, presumably due to loss of Hsp104 function.

The drop in viability observed in our *tps1Δhsp104Δ* mutant after short-term desiccation was significant but small compared to the desiccation sensitivity observed in our previous studies for wild-type exponential cells [16]. This result suggests that trehalose and Hsp104 are only partially responsible for the short-term desiccation tolerance of stationary cells under our standard laboratory conditions of desiccation. We wondered whether their cooperation in protecting desiccated cells might become more crucial beyond our standard assay conditions. With this in mind, we examined the ability of different yeast strains to survive when desiccated and then challenged with heat stress, a condition yeast cells might commonly experience in the wild.

We began by comparing the survival of a saturated culture of wild-type yeast in liquid and after desiccation when exposed to 30 min of elevated temperatures. As expected, wild-type cells in liquid culture lose almost all viability at temperatures above 52°C (Figure 4A). Remarkably, desiccated wild-type cells are extremely heat tolerant, with loss of viability beginning only after heating cells at 72°C, and more than 0.1% of cells surviving above 80°C (Figures 4A and 4B). Both *tps1Δ* and *hsp104Δ* mutant strains behaved similarly to wild-type, remaining viable after 30 min of heat shock at temperatures up to 72°C and losing viability slowly with

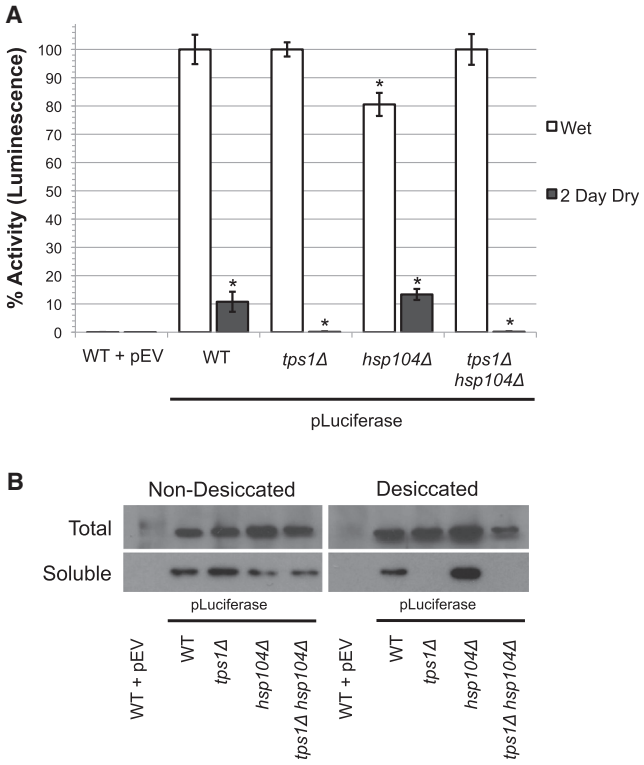

Figure 5. Desiccation Induces Proteotoxic Stress

Luciferase activity as a reading of proteostasis of stationary and desiccated cells.

(A) WT, *tps1Δ*, *hsp104Δ*, and *tps1Δhsp104Δ* strains were transformed with a temperature-sensitive firefly luciferase-fusion protein from the constitutive glyceraldehyde-3-phosphate (GPD) promoter (p426-GPD-FFL) or empty vector (pEV, p426-GPD) and were grown to saturation in media lacking uracil. Desiccated samples were air-dried for 2 days followed by rehydration in YPD or SC-HIS + cycloheximide (10 μl/ml, to block new FFL protein synthesis). Luciferase activity was measured in vivo by addition of 0.5 mM D-Luciferin to equal number of intact cells. Light emission was measured immediately with a luminometer. Percentage of activity was normalized to WT saturated "wet" sample. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 2 day dry sample compared with the corresponding wet sample.

(B) Cells were prepared as in (A). Total cellular protein was extracted, and aggregated proteins were sedimented by high-speed centrifugation. Total protein (total) and high-speed supernatants (soluble) were then followed by SDS-PAGE and reacted with antiserum recognizing luciferase.

increasing temperature (Figure 4B). The viability of desiccated strains unable to synthesize trehalose and Hsp104 simultaneously (*tps1Δhsp104Δ*) was reduced almost three orders of magnitude at the initial 52°C tested. These cells maintained a constant, albeit low level of viability at increasing temperatures, yet were completely unable to survive temperatures above 85°C. These results indicate that trehalose and Hsp104 cooperate to mitigate the combined lethal damage of desiccation and extreme heat stress.

Desiccation Induces Proteotoxic Stress that Is Mitigated by Trehalose

The overlapping functions of trehalose and Hsp104 in short-term desiccation tolerance added to the observations that implied a potential role of trehalose in mitigating desiccation-induced proteotoxicity. However, a direct measure of the proteotoxicity of desiccation has not been reported. To assess desiccation-induced proteotoxicity, we took advantage of firefly luciferase (FL), an established and very sensitive reporter for protein misfolding/aggregation [23, 32]. Briefly, we transformed wild-type and different mutant strains with a plasmid directing constitutive expression of a temperature-sensitive FL protein. FL activity can be determined by monitoring light emission in intact, living cells.

Luciferase emission was high in all strains in saturated cultures (Figure 5A). In contrast, when cells were desiccated for 2 days, rehydrated, and immediately assayed for luciferase activity, all desiccated strains had a dramatic loss of luminescence. Strains lacking the ability to produce trehalose (*tps1Δ*, *tps1Δhsp104Δ*) exhibited no detectable FL activity after desiccation, compared to wild-type and *hsp104Δ* (Figure 5A). This result demonstrated that desiccation can dramatically perturb protein folding, and this perturbation is further exacerbated by the lack of trehalose.

To complement the luminescence assay, we employed differential centrifugation as an independent method to assess potential aggregation of FL. FL extracted from stationary phase cultures was stable and remained largely soluble in all strains tested (Figure 5B). In stark contrast, desiccated strains lacking trehalose exhibited clear protein aggregation, as evidenced by a lack of FL in the soluble fraction. The lack of FL in the soluble fraction in these strains is not the result of protein degradation, because total protein levels for FL remained constant (Figure 5B). We conclude that desiccation, a naturally occurring environmental condition, is a potent protein denaturant, and desiccation-mediated denaturation of at least this reporter is substantially mitigated by trehalose. We also examined the abundant soluble proteins in our differential centrifugation assay. Desiccation did not change the solubility of the abundant soluble proteins in any of our strains. This result suggests that desiccation promotes aggregation of a subset of proteins like luciferase that are prone to aggregation.

As a simple test of this idea, we used prion propagation as a second readout to assess the effects of desiccation and trehalose on proteostasis. A prion is a misfolded form of a protein that propagates itself by associating with newly synthesized forms of that protein, converting them to the misfolded state [34]. Once misfolded, prions often aggregate, in some cases forming amyloid-like deposits. The tendency of prions to aggregate must be tempered so that some prions exist as soluble prion seeds. These seeds act as epigenetic agents of heritability by randomly diffusing into daughter cells during cell division. Given these features of prions, defects in prion propagation could reflect a number of changes in proteostasis, for example, changes in the protein folding milieu that favor reversion of the prion to the wild-type state, or in protein solubility such that prion seeds fail to remain soluble.

With this in mind we asked whether desiccation impacted proteostasis by monitoring the propagation of [*PSI*$^+$] and [*GAR*$^+$], two well-characterized prions in yeast with molecularly distinct modes of propagation [35–37]. [*PSI*$^+$] is a cytoplasmic prion of the translation termination factor Sup35 and requires Hsp104 for its propagation [35]. [*GAR*$^+$] is a membrane-associated heterotypic conformer of the plasma membrane proton pump Pma1 and the glucose-repressed gene regulator Std1 and requires Hsp70 proteins for its propagation [37]. Their propagation can be easily followed by convenient phenotypic reporters: growth on adenine (ADE$^+$) for [*PSI*$^+$] and growth on a nonfermentable carbon source in the

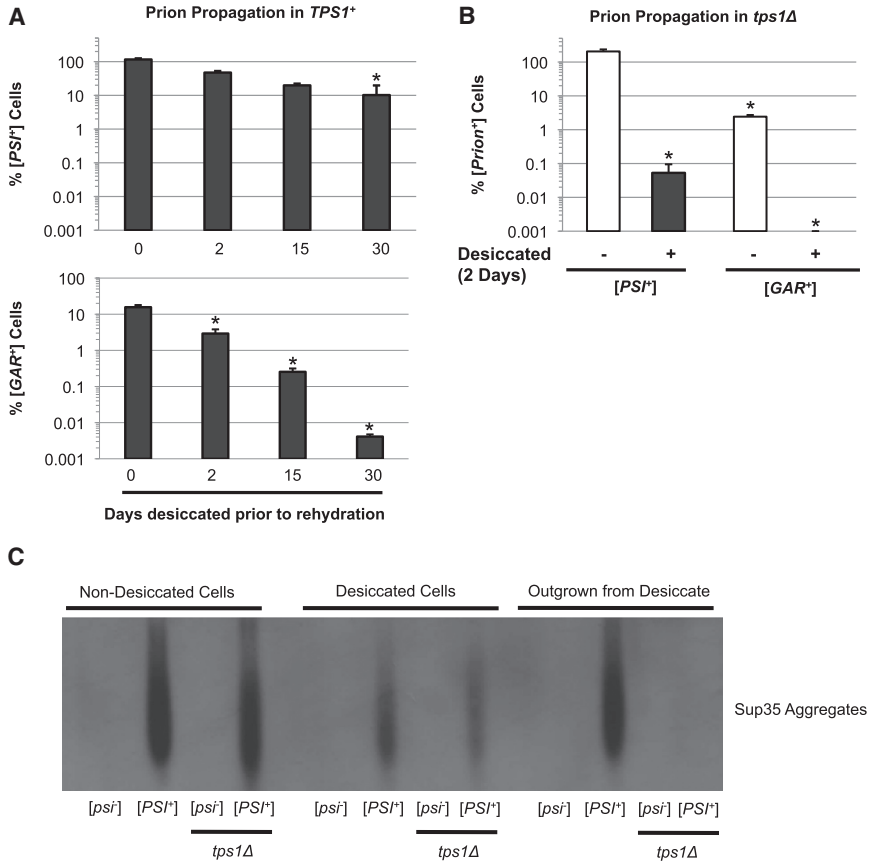

Figure 6. Trehalose Is Important for Prion Propagation during Desiccation

Prion propagation as a measure of protein propagation after desiccation.

(A) WT strains harboring either [*PSI*$^+$] or [*GAR*$^+$] were prepared and analyzed as described for desiccation tolerance assay and rehydrated after 2, 15, and 30 days. To assess prion state, cells were plated on media lacking adenine (SC-ADE) for [*PSI*$^+$] strains, or on YP media with 0.05% glucosamine and 2% glycerol for [*GAR*$^+$]. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 2, 25, or 30 day dry sample compared with the corresponding "wet" sample.

(B) *tps1Δ* strains harboring either [*PSI*$^+$] or [*GAR*$^+$] were prepared and analyzed as described for desiccation tolerance assay and rehydrated after 2 days; prion state was measured as described for (A) in media with galactose instead of glucose. Asterisk represents the statistical significance ($p \leq 0.005$, t test) of the 2 day dry sample compared with the corresponding "wet" sample.

(C) WT and *tps1Δ* strains with or without the [PSI$^+$] prion were assayed by SDD-AGE. Total cellular protein was extracted followed by SDD-AGE and reacted with antiserum recognizing Sup35. Extracts were taken from saturated nondesiccated cells, desiccated cells, and desiccated cells rehydrated and grown to saturation (outgrown).

presence of glucosamine (GLU^R) for [*GAR*$^+$]. If a cell containing [*PSI*$^+$] or [*GAR*$^+$] is able to propagate the prion, it will give rise to a colony of clonal descendents that is ADE$^+$ or GLU^R, respectively. By this assay, the percentage of [*PSI*$^+$] cells that could propagate the prion after desiccation was found to decline a few fold after 2 days of desiccation and 10-fold upon extended desiccation (Figure 6A). The percentage of [*GAR*$^+$] cells that could propagate their prion also declined a few fold after 2 days of desiccation but nearly 1,000-fold after 30 days (Figure 6A). Thus, long-term desiccation perturbed the propagation of two different prions with independent nucleation and propagation mechanisms. These results suggest that desiccation perturbs a common property of proteostasis needed for prion propagation. In addition, the correlation between time-dependent loss of prions and time-dependent loss of trehalose is consistent with a role for trehalose in facilitating prion propagation.

To test whether trehalose levels affect the propagation of prions, we compared [*PSI*$^+$] and [*GAR*$^+$] propagation in wild-type and *tps1Δ* strains. Both wild-type and *tps1Δ* cells in saturated cultures prior to desiccation were equally competent to propagate [*PSI*$^+$]. After only 2 days of desiccation, cells lacking trehalose were 200-fold less competent to propagate [*PSI*$^+$] compared to wild-type (Figure 6B). Lack of trehalose had an even more severe effect on [*GAR*$^+$]. The percentage of cells in saturated cultures able to propagate [*GAR*$^+$] was reduced 10-fold in saturated cultures of *tps1Δ* compared to wild-type. Upon desiccation none of the [*GAR*$^+$] cells were able to propagate [*GAR*$^+$] (Figure 6B). Thus [*GAR*$^+$] propagation is even more perturbed by desiccation and more dependent upon trehalose to survive desiccation than [*PSI*$^+$]. The dramatic requirement for trehalose for the propagation of both [*PSI*$^+$] and [*GAR*$^+$] strongly suggests that trehalose modulates some aspect(s) of proteostasis necessary for prion propagation during desiccation.

What is the proteotoxic stress that is perturbing prion propagation upon desiccation and mitigated by trehalose? Desiccation could cause an unusual environment that promotes refolding of prions to their proper state, but this refolding is blocked by trehalose. In this scenario, prion aggregates should be detectable in wild-type and *tps1Δ* cells prior to desiccation but absent in *tps1Δ* cells immediately after rehydration. Alternatively, desiccation could cause an environment favoring hyperaggregation that blocks or inhibits prion seed formation. In this scenario, prion aggregates should be present in both wild-type and *tps1Δ* cells immediately after rehydration. However, in *tps1Δ* cells, the inability to solubilize prion seeds would block prion inheritance during subsequent cell divisions, and the prion aggregates should disappear from progeny cells with outgrowth.

To test these two possibilities, we followed the [*PSI*$^+$] aggregates in wild-type and *tps1Δ* strains by SDD-AGE analysis before desiccation, immediately after rehydration, and after outgrowth of the rehydrated cells. Similar amounts of aggregates were detectable in both strains prior to desiccation, as expected. Immediately after rehydration, the levels of aggregate were also similar between wild-type and *tps1Δ* strains (Figure 6C). The slight reduction in detectable prion aggregates in both wild-type and *tps1Δ* strains immediately after desiccation may reflect difficulty in recovering aggregates from desiccated cells. Importantly, upon outgrowth, the aggregates were lost from the *tps1Δ*, but not the wild-type, strain (Figure 6C). Taken together, our results suggest that desiccation causes extensive protein aggregation of prions that is

mitigated by trehalose. In the absence of trehalose, excessive aggregation of prions prevents prion seed formation, thereby blocking prion propagation.

Discussion

Numerous previous studies have correlated high and low levels of trehalose with desiccation tolerance and sensitivity, respectively. Here, we show that trehalose is essential for survival to long-term desiccation while cooperating with Hsp104 for survival to short-term desiccation. Recently, a role for trehalose in desiccation tolerance was also shown in *Caenorhabditis elegans* dauers and the mosquito *Anopheles gambie*, a major vector for *Plasmodium falciparum* [19, 20]. Thus, a causative role for trehalose in desiccation tolerance has now been established across taxa. The change in trehalose concentration during long-term desiccation and return to growth allowed us to perform the first in vivo titration curve of desiccation tolerance and trehalose concentration. We observe a step-like function. Even small reductions in trehalose concentration (from 300 to 100 μg/ml) fail to promote robust tolerance. Knowledge of this threshold concentration for physiological-effective trehalose will be an important metric to assess the in vivo relevance of the many in vitro activities of trehalose.

One of the most remarkable properties of anhydrobiotes is their ability to remain viable for extended periods of desiccation. Here, we provide three lines of evidence that trehalose is essential for yeast's ability to survive long-term desiccation. First, the natural slow decay in trehalose concentration after desiccation correlates with a slow decrease in desiccation tolerance. Second, cells lacking trehalose (*tps1Δ*) are greatly compromised for long-term desiccation tolerance. Finally, inactivation of the trehalases greatly slows down the decline in trehalose concentration in desiccated cells and dramatically improves their long-term desiccation tolerance. Because sustaining trehalose levels is essential to survival of long-term desiccation, stresses from desiccation must persist or continue to accrue during desiccation. One of these stresses is likely protein aggregation as evidenced by decreased propagation of prions with time of desiccation (this study).

The ability of trehalases to breakdown trehalose in desiccated cells is remarkable. Trehalases must be active in cells with <5% water. This result suggests that not all metabolic activity of yeast is completely ablated by desiccation. It should be noted that this metabolic activity is extremely slow. In desiccated cells, these trehalases reduce trehalose concentrations only 10-fold over 6 months. In contrast, when desiccated cells are rehydrated, the trehalases reduce trehalose 1,000-fold within hours. Nonetheless, the slow metabolism may allow damage from desiccation to accrue leading to decreased long-term survival. An interesting question is why yeast, an anhydrobiote, has trehalases if they compromise long-term survival to desiccation. The elevated trehalose that protects against desiccation can also act as a potent storage carbohydrate providing a ready energy source when water returns. Upon rehydration, cells with trehalases can rapidly convert trehalose to glucose and win the arms race for growth. Thus, the level of trehalases may have to be optimized in anhydrobiotes, sufficiently low to prevent trehalose metabolism and support long-term survival to desiccation, but sufficiently high to allow trehalose metabolism and rapid growth upon rehydration.

A second important insight comes from our analyses of short-term desiccation tolerance. We show that the *tps1Δhsp104Δ* mutant, but not *tsp1Δ* or *hsp104Δ*, is sensitive to short-term desiccation and hypersensitive to a secondary heat shock, a likely frequent event in natural habitats. These results demonstrate that Hsp104 plays a role in desiccation tolerance and has overlapping functions with trehalose in desiccation tolerance. This redundancy is reminiscent of their overlapping function in heat shock [23].

If Hsp104 can perform trehalose's function, then why do desiccation-tolerant yeast cells make both molecules? We provide two answers to this question. First, we show that trehalose promotes the propagation of both a cytoplasmic prion [*PSI*$^+$] and a membrane prion [*GAR*$^+$], whose propagation in culture normally require Hsp104 and Hsp70 chaperones, respectively [35, 37]. This result indicates that trehalose is a more versatile stress effector than Hsp104 in proteostasis. We also show that Hsp104 is not required for long-term-desiccation tolerance nor does it help trehalose to promote it. We suggest desiccated cells initially contain active protein chaperones and trehalose. However, with time, the activities of protein chaperones are compromised because of their complex structure and requirement for energy. In contrast, a small molecule stress effector like trehalose is stable and energy independent. Thus, the stability, energy independence, and functional versatility of trehalose make it particularly better suited than protein chaperones to protect yeast against the vagaries experienced during long-term desiccation.

The link between trehalose, Hsp104, heat shock, and now desiccation infers a role for trehalose in protecting desiccated cells from proteotoxicity. We provide the first evidence that desiccation does indeed alter proteostasis by showing that desiccation leads to inactivation of a luciferase reporter and an inability to efficiently propagate a membrane and a cytoplasmic prion. Having established that desiccation induces proteotoxicity, we provide evidence that this is likely due to hyperprotein aggregation that is mitigated by trehalose. We show that prion propagation is dramatically reduced by desiccation when trehalose levels are compromised either by long-term desiccation or by inactivation of trehalose biosynthesis. Furthermore, we show that desiccation does not block prion propagation by eliminating the prion from the desiccated cell but rather prevents its inheritance to daughter cells upon rehydration, likely because of hyperaggregation. Interestingly, desiccation does not induce detectable insolubility of the abundant soluble proteins, suggesting that Hsp104 and trehalose promote desiccation tolerance by acting on a subset of cellular proteins, which like prions and luciferase are prone to insolubility. Identifying these target proteins will be an important next step.

How does trehalose block aggregation? Because trehalose is a potent energy source when it is broken down into glucose, it has been difficult to differentiate in vivo whether trehalose acts directly as a stress effector or simply provides an energy source for other energy-dependent stress effectors like protein chaperones. However, the latter seems very unlikely given that desiccation tolerance is enhanced by inactivation of trehalases that are necessary for the breakdown of trehalose to glucose. Therefore, we favor a model in which trehalose directly protects against desiccation-induced aggregation, providing the first in vivo evidence for one of the many activities of trehalose that has been suggested from in vitro experiments. We favor the notion that the antiaggregation activity of trehalose derives from its ability to vitrify, but we cannot eliminate other possibilities. One possible way to distinguish between these scenarios will be to reexamine trehalose's

biochemical and biophysical activities over the narrow concentrations that we define here as critical for differentiating between desiccation sensitivity (100 μg/ml) and tolerance (300 μg/ml).

In summary, the demonstration of trehalose activity in this study of desiccation tolerance in budding yeast has provided important insights into its function in long-term desiccation tolerance, its cooperation with Hsp104, and its in vivo versatility as a chemical chaperone to protect against the toxicity of protein aggregation of membrane and cytoplasmic proteins. It will be interesting to assess whether trehalose protects cells against other desiccation induced stresses, and whether long-term survival to desiccation requires other factors correlated with desiccation tolerance like LEA proteins.

Experimental Procedures

Detailed experimental procedures are presented in the Supplemental Information.

Desiccation Tolerance Assay

Approximately 10^7 cells were withdrawn from liquid cultures and washed twice in dilute PBS (1/8× PBS) and then brought to a final volume of 1 ml. Undesiccated controls were plated for colony counting. Two hundred microliter aliquots were then transferred to a 96-well tissue culture plate (Becton Dickinson, 353075) and centrifugated, and water was removed without disturbing the cell pellet. Cells were allowed to desiccate in a 23°C incubator with a constant 60% relative humidity (RH), with the lid raised, for at least 48 hr. Long-term desiccation experiments were kept for indicated time periods in a 96-well tissue culture plates at 23°C, 60% RH. Samples were resuspended in assay buffer and plated for colony counting. Data were entered into a spreadsheet (Microsoft Excel 2008 for Mac version 12.3), and the number of colony forming units per milliliter (cfu/ml) for each plate was computed. For each experiment, cfu/ml for the two controls was averaged. The relative viability of each of the two experimental samples was determined by dividing the cfu/ml for that sample by the average cfu/ml of the control plates. These two relative viability values were then averaged using the AVE worksheet function, and their SD was computed using the STDEV worksheet function (experimental workflow in Figure S3A).

Trehalose Assay

Trehalose concentration assays were performed as described previously with minor modifications [38]. Approximately 10^7 cells were withdrawn from liquid cultures (data presented as μg/ml equals μg/10^7 cells). Samples were washed with ice-cold H_2O and resuspended in 250 μl 0.25 M Na_2CO_3 and stored at −80°C until processed. Samples were boiled at 95°C–98°C for 4 hr followed by addition of 150 μl 1 M acetic acid and 600 μl 0.2M sodium acetate. For controls, half of each sample was transferred to a new tube, and the remaining half was incubated with 0.025 U/ml trehalase (EC 3.2.1.28; Megazyme) at 37°C overnight. Samples were centrifugated at 14,000 rpm in a microcentrifuge for 3 min and assayed for glucose using a Glucose Assay kit (GAGO20; Sigma-Aldrich). Briefly, 200 μl of test sample was transferred to a fresh tube. Four hundred microliters of the assay reagent was added into each tube to start the colorimetric reaction. After a 30 min incubation at 37°C, 400 μl of 12N H_2SO_4 was added to stop the reaction. Absorbance at 540 nm was determined to assess the quantity of glucose liberated from trehalose.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.10.005.

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